Material and Methods

hiPSCs expansion and differentiation.

Human iPSC lines BC1 (from Dr. Linzhao Cheng (JHU)), T1D H2.1 (from Dr. Shuibing Chen (Weill Cornell Medical College)), and T1D 1018S (from Dr. Egli Dietrich (Columbia University)) were cultured as previously described.[1,2,3] ell lines were routinely examined for pluripotent markers using immunofluorescence staining and flow cytometry analysis for TRA-1-60, TRA-1-81, SSEA4, and Oct4. Human iPSCs (T1D H2.1 and 1018S or BC1) were collected through digestion with ethylenediaminetetraacetic acid (EDTA; Promega, Madison, WI), separated into an individual cell suspension using a 40 µm mesh strainer (BD Biosciences), and plated onto collagen IV (Trevigen) coated plates at a concentration as follows. Original differentiation condition at $5x10^4$ cells/cm^2. Optimized conditions: (i) increased cell seeding density condition at $1x10^5$ cells/cm^2; (ii) ROCK suppression condition at $5x10^4$ cells/cm^2 with 10µM ROCK inhibitor Y-27632 (Stemcell Technologies). During mesodermal induction, cells were cultured in a differentiation medium composed of alpha-MEM (Invitrogen Carlsbad, CA), 10% FBS (Hyclone) and 0.1 mM β-mercaptoethanol (β-ME) as previously described. In early vascular differentiation, cells were cultured in endothelium growth medium (Promocell) supplemented with 50ng/mL VEGF (R&D) and SB-431542 (Sigma-Aldrich, St. Louis, MO), thereafter, called high VEGF media.[4]

Flow cytometry

Flow cytometry was performed as previously described.[4] Briefly, cells were incubated with FITC- or PE-conjugated antigen specific antibodies for markers outlined in the text (supplementary table 1). All analyses were done using corresponding isotype or no stain controls. Forward-side scatter plots were used to exclude dead cells. User guide instructions were followed to complete the flow cytometry analysis via CellQuest Pro software (BD Biosciences).

Immunofluorescence and imaging (2D and 3D)

Cells were prepared for immunofluorescence as previously described.[4] Briefly, cells were fixed using 3.7% paraformaldehyde for 30 min at room temperature and washed three times using PBS. The fixed cells were permeabilized with 0.1% Triton X-100 for 20 min and incubated with 1% BSA blocking solution at room temperature for 1 hour. Samples were incubated with either the antigen specific primary antibodies for the markers outlined in the text, followed by an appropriate secondary antibodies (Supplementary Table 1), or with phalloidin (1:500; Molecular Probes) and DAPI (1:10,000; Molecular Probes). Both primary and secondary antibodies were diluted in antibody diluent (DAKO). The immunolabeled cells were examined using a fluorescent microscope (Olympus BX60) and imaged using a confocal microscope (Zeiss LSM 700 and LSM 780). Confocal z-stacks of 3D vascular networks were analyzed using Imaris 8.0 Filament software (Bitplane).

Matrigel cord formation

Cord formation on Matrigel was assessed as previously described.[4,5] Briefly, Matrigel was cast into 16 well chamber slides (Lab-Tek). After polymerization, 15,000 cells were seeded per well in high VEGF media. Cord formation was observed after 4, 6 and 12 hours

Tumor necrosis factor alpha (TNFα) response

T1D-derived early ECs were magnetically sorted using VEcad-PE (Miltenyi Biotech). Sorted ECs were culture on in high VEGF media for an additional 6 days to mature. On the last day of maturation (day 18), high VEGF media was removed and sorted ECs were treated with 10ng/mL TNFα in high VEGF media (Promocell) for 24 hours. The treated ECs were washed with PBS before dissociated to analyze ICAM-1-FITC (Sigma) expression using flow cytometry.

Acrylated hyaluronic acid (AHA) hydrogel and EVCs constructs
AHA hydrogels were prepared as previously reported.[4, 6, 7, 8] Briefly, AHA (3 wt%) was dissolved in a triethanolamine buffer solution (TEOA buffer, Sigma). The cell adhesive peptides (RGDS; GenScript) were dissolved in TEOA buffer and added to the AHA solution at final peptide concentrations of 3.7 mM (corresponding to 10% of available acrylate groups within 3 wt% AHA) and were allowed to react for one hour with gentle shaking. Recombinant human $VEGF_{165}$ (R&D), bFGF (R&D), Ang-1 (R&D), tumor necrosis factor-alpha (TNF-α; R&D) and stromal cell-derived factor-1 (SDF-1; R&D) were added at 50 ng/mL into the AHA-RGDS mixture. Following the resuspension of cells into this solution, MMP peptide crosslinker (MMP; GenScript) dissolved in TEOA buffer was added at 4.83 mM (corresponding to the 25% of available acrylate groups within the 3 wt% AHA). Derived EVCs were encapsulated in HA hydrogels at a density of $4x10^6$ cells/mL and cultured for up to 3 days in endothelial growth media 2 (EGM2; Lonza). Visualization and image acquisition were performed using an inverted light microscope (Olympus) and confocal microscope (Zeiss LSM 700 and 780) along the culture.

Hypoxia-inducible (HI) hydrogels and EVCs constructs
HI hydrogels were prepared as previously reported.[9] Briefly, gelatin and ferulic acid polymer solution (4 wt%) was dissolved in PBS (pH 7.4) and mixed with EVC pellets to provide cell suspension, and then laccase solution (100 U/mL) was added at a volume ratio of 3:1 (polymer solution:laccase solution) and gently mixed for 1 min at 37°C. The final concentration of the polymer, laccase and cells were 3 wt%, 25 U/mL and $3x10^6$cells/mL, respectively. To generate hypoxic and non-hypoxic hydrogels, 45µL and 90µL cell mixture was added respectively to each well of a 96-well plate and allowed to react at 37°C for 20 min. On top of the EVC encapsulated hypoxic and non-hypoxic hydrogels, endothelial cell growth media (Lonza) and a growth factor mix as described above for AHA hydrogels at 10ng/mL was added at 200µL and 100µL, respectively. EVC encapsulated hydrogels were cultured under standard cell culture conditions (37°C and 5% CO_2) for up to 72 hours. The culture medium was replaced every 24 hours. EVC morphologies were observed using optical microscopy (in phase-contrast mode) and confocal microscopy (Zeiss LSM 780).

Zebrafish xenograft model and image analysis. The zebrafish xenograft model was adapted from Orlova et al, 2014[10] and described as follows. Fertilized zebrafish embryos were collected and sorted 24 hours post breeding. GFP (Tg(flk1:GFP))-expressing embryos were dechorionated and separated from GFP-negative embryos by fluorescence microscope. At approximately 48 hours-post-fertilization (h.p.f.), hiPSC-derived EVCs were injected into the zebrafish blood vessels via the Duct of Cuvier (DC). Prior to injection, differentiated EVCs were stained with PKH26 fluorescent cell linker (Sigma). Approximately 100-150 cells were injected into each embryo. On the same day of injection, the injected embryos were examined to confirm that the injected cells were present in the circulation. Live or fixed embryos were examined 5 days post injection to examine for integration using fluorescence microscope (Olympus) and confocal microscopy (Zeiss LSM 780). A total of 6 embryos injected with H2.1 T1D-EVCs and a total of 6 embryos injected with 1018S T1D-EVCs were randomly selected from at least 3 different breeding pairs for acquiring high quality confocal Z-stacks. Three dimensional confocal stacks of the injected zebrafish embryos were analyzed using Imaris software (Bitplane). Total number of the injected cells present in the zebrafish embryos were counted using spot analysis for spots that are larger than 10µm (equivalent to the average EVC cell size). These spots were then co-localized to the endogenously expressed-GFP in the endothelial cells of the zebrafish embryos. The final analysis was done by taking the percentage of total co-localized EVCs versus total EVCs present in the zebrafish embryos.

Statistics

All analyses were performed in triplicate samples for at least n=3. Two-tailed t test was performed to determine significance. All graphs were drawn using GraphPad Prism 5. Significance levels were set at *p<0.05, **p<0.01, and ***p<0.001.

References:

1. Chou BK, Mali P, Huang X, Ye Z, Dowey SN, Resar LM, Zou C, Zhang YA, Tong J, Cheng L. Efficient human ips cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. *Cell Res*. 2011;21:518-529

2. Maehr R, Chen S, Snitow M, Ludwig T, Yagasaki L, Goland R, Leibel RL, Melton DA. Generation of pluripotent stem cells from patients with type 1 diabetes. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106:15768-15773

3. Johannesson B, Sagi I, Gore A et al. Comparable frequencies of coding mutations and loss of imprinting in human pluripotent cells derived by nuclear transfer and defined factors. *Cell Stem Cell*. 2014;15:634-642

4. Kusuma S, Shen YI, Hanjaya-Putra D, Mali P, Cheng L, Gerecht S. Self-organized vascular networks from human pluripotent stem cells in a synthetic matrix. *Proceedings of the National Academy of Sciences of the United States of America*. 2013;110:12601-12606

5. Vo E, Hanjaya-Putra D, Zha Y, Kusuma S, Gerecht S. Smooth-muscle-like cells derived from human embryonic stem cells support and augment cord-like structures in vitro. *Stem Cell Rev*. 2010;6:237-247

6. Hanjaya-Putra D, Bose V, Shen YI, Yee J, Khetan S, Fox-Talbot K, Steenbergen C, Burdick JA, Gerecht S. Controlled activation of morphogenesis to generate a functional human microvasculature in a synthetic matrix. *Blood*. 2011;118:804-815

7. Khetan SKJ, Burdick JA. Sequential crosslinking to control cellular spreading in 3-dimensional hydrogels. *Sofr Matter*. 2009;5:1601-1606

8. Khetan S, Burdick JA. Patterning network structure to spatially control cellular remodeling and stem cell fate within 3-dimensional hydrogels. *Biomaterials*. 2010;31:8228-8234

9. Park KM, Gerecht S. Hypoxia-inducible hydrogels. *Nature communications*. 2014;5:4075

10. Orlova VV, van den Hil FE, Petrus-Reurer S, Drabsch Y, Ten Dijke P, Mummery CL. Generation, expansion and functional analysis of endothelial cells and pericytes derived from human pluripotent stem cells. *Nature protocols*. 2014;9:1514-1531